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Original Article

Comparison of the effects of enamel matrix derivative and mineral trioxide aggregate on the mineralization potential of human cementum-derived cells

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Abstract *Background/purpose:* Cementogenesis is a critical event in the repair of endodontic perforations and the enamel matrix derivative (EMD) was reported to induce cementogenesis when used in periodontal disease. Yet, there is no study investigating the effects of EMD compared with mineral trioxide aggregate (MTA), a well-known material used for perforation repair. Because cementoblasts play important roles in the repair and regeneration of cementum, the aim of this study was to investigate the effect of EMD on the mineralizing potential of human cementum-derived cells (HCDCs).
Materials and methods: HCDCs were cultured as previously described. ProRoot MTA was mixed and placed on the bottom of 6-well tissue culture plate inserts under aseptic conditions. These were allowed to set for 24 hours in a humidified incubator at 37°C. Cells were seeded onto culture plates at an initial density of 5000 cells/cm^2 and were treated with either MTA or EMD. Cells with only culture medium were used as the control. Levels of osteopontin and matrix metalloproteinase-13 were assessed by an enzyme-linked immunosorbent assay with supernatants of the culture media collected on Days 3 and 6 of incubation. Mineralization was detected on Day 6 by Alizarin red staining of cells.
Results: Concentrations of osteopontin in the EMD group were significantly higher ($P < 0.05$) than those in the control group at all time points. On both Days 3 and 6, there was an increase in matrix metalloproteinase-13 expression in the EMD group compared with the control group, but the difference was significant only on Day 6 ($P < 0.05$).

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Conclusions: Compared with MTA, EMD significantly stimulated the mineralization potential of HCDCs, which implies the potential of EMD for use as an endodontic repair material.

Introduction

Root perforation is an undesirable incident that can occur at any stage of root-canal therapy and in such cases cementogenesis is a critical event for regenerating periodontal tissues.[1] Mineral trioxide aggregate (MTA) has been the material of choice for perforation repair since its introduction in the early 1990s. MTA is known to create a biocompatible environment in periodontal tissues and induce cementogenesis when used in a perforated area.[2] However, MTA causes inflammatory and necrotic changes in subjacent tissues under the process of hard tissue formation, which implies the need to develop more nontoxic and biologically active agents.[3]

During tooth development, cells of Hertwig's epithelial root sheath secrete enamel matrix derivative (EMD) onto the newly formed root dentin surface.[4] Experimental applications of EMD on the denuded root dentin surface of human and monkey teeth resulted in the formation of a new layer of acellular cementum and the formation of alveolar bone.[5] *In vitro* studies also support the capacity of EMD to stimulate cementoblastic activity, which suggests the possibility of its application in perforation repair.[6]

Among many mineralization markers, osteopontin (OPN) is one of the major noncollagenous proteins in the root cementum and other mineralized tissues.[7] It is an adhesion molecule of the extracellular matrix (ECM) of calcified tissues and is related to wound healing, inflammation, and immunologic responses.[8] In human and monkey periodontal regeneration studies, OPN expression was demonstrated at the border near newly formed cementum and bone.[9] Matrix metalloproteinase (MMP)-13, also called collagenase III, is a member of the family of MMPs produced in high amounts by cells with mineralizing potential. It was reported to be involved in degradation and remodeling of the ECM.[10]

Most previous studies on cementoblasts with MTA or EMD used murine cementoblasts. Recently, a reliable method was developed to culture cells from human cementum.[11] To the best of our knowledge, there are no studies comparing the effects of MTA and EMD on human cementum-derived cells (HCDCs). The aim of the present study, therefore, was to investigate the effects of EMD on the mineralization capacity of HCDCs and evaluate its potential as an endodontic repair material.

Materials and methods

Cell culture

HCDCs were isolated and characterized as previously described.[11] Premolars freshly extracted for orthodontic reasons were used. Informed consent was acquired in accordance with the Helsinki Declaration and the study was approved by the Ethics Committee of Asan Medical Center (Seoul, Korea). Teeth were either kept in serum-free Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, Buffalo, NY, USA) containing 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco) overnight at 4°C or immediately processed after extraction. The periodontal ligament was manually dissected from the tooth root with a surgical scalpel. After extensive washing with plain medium, teeth were subjected to collagenase P (100 mU/mL; Boehringer-Mannheim, Mannheim, Germany) treatment of 1.5–2 hours at 37°C. The medium with released cells was discarded and the cementum, together with a thin layer of underlying dentin, was dissected and collected. These fragments were thoroughly washed and then minced with scissors until small chips (<0.5 mm in diameter) were obtained. The chips were next digested again with collagenase P for 1.5 hours at 37°C to remove all cells unprotected by mineral. After this step, the fragments were thoroughly washed with the medium and placed in 150-mm plastic tissue culture Petri dishes (Costar, Cambridge, MA, USA) containing growth medium [DMEM/F12 supplemented with penicillin/streptomycin and 10% fetal bovine serum (Gibco)]. The cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. The medium was changed every other day. Under the conditions used (i.e., plating chips derived from one tooth placed into a 150-mm plate), HCDCs formed colonies that were separated by large distances. When the cell number within a colony reached 400–500 (usually after approximately 4 weeks of culture), cells from individual colonies were scraped off with a Pasteur pipette, transferred to wells of 12-well tissue culture plates (Costar), and cultured in growth medium until semiconfluent. HCDCs were subcultured and cells from passages 8 and 9 were used in the experiment.

Preparation of test materials

Under aseptic conditions, MTA (ProRoot; Dentsply, Tulsa, OK, USA) was mixed according to the manufacturer's instructions and placed on the bottom of culture plate inserts (Millicell, Millipore, Bedford, MA, USA) of six-well culture plates, which had a membrane pore diameter of 0.4 μm. They were allowed to set for 24 hours in a humidified incubator at 37°C. They were then rinsed three times with 70% ethanol and immersed in serum-free DMEM (2 mL/well) for an additional 72 hours. Emdogain-gel (30 mg/mL) (Straumann, Basel, Switzerland), a commercial preparation of porcine fetal EMD, was used as the EMD. The EMD was diluted with sterile distilled water to a concentration of 100 μg/mL.[12] Cells were seeded onto six-well culture plates at an initial density of 5000 cells/cm^2. After 24 hours of incubation, cells were subjected to two different treatments. In Group 1, EMD was directly added to the culture

medium. In Group 2, the prepared MTA on the insert was placed into the culture medium. Cells with only culture medium were used as the control.

Enzyme-linked immunosorbent assay

After 24 hours of initial attachment, the medium was switched to mineralizing medium in which DMEM containing 2% fetal bovine serum was supplemented with ascorbic acid (50 μg/mL) and β-glycerol phosphate (10 mmol/L). The mineralizing medium was changed on Day 3 and cells in Group 1 were restimulated with EMD to ensure that EMD was continually present in the medium throughout the experimental period. Supernatants of the culture medium of each group were collected on Days 3 and 6. MMP-13 levels were measured with an Amersham MMP-13, Human, Biotrak ELISA System (GE Healthcare, Buckinghamshire, UK) and OPN levels were determined by a Quantikine Human OPN Immunoassay Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. A microplate reader (Spectra Max 340, Sunnyvale, CA, USA) was used to measure the absorbance at 450 nm.

Alizarin red staining

Alizarin red staining is specific for calcium deposition.[13] Mineralization on Day 6 was assessed by staining with Alizarin red (Sigma-Aldrich, St Louis, MO, USA). Alizarin red at 40 mmol/L was prepared in distilled water and adjusted to pH 4.2 with ammonium hydroxide. It was then applied to cells in the six-well plates for 30 minutes at room temperature with gentle agitation. Then, cells were rinsed with distilled water and allowed to dry.

Statistical analysis

The statistical analysis of the data used one-way analysis of variance followed by Scheffe's test. Statistical significance was accepted at $P < 0.05$.

Results

Enzyme-linked immunosorbent assay

OPN levels of the MTA group were higher than those of the control group on both Days 3 and 6, but the difference was significant only on Day 6 ($P < 0.05$). The concentration of OPN in the EMD group was significantly higher ($P < 0.05$) than that in the control group at each time point (Fig. 1).

On both Days 3 and 6, there were increases in MMP-13 expression in the EMD group compared with the control group, but the difference was significant only on Day 6 ($P < 0.05$). MMP-13 was also upregulated in the MTA group, however the difference from the control group was not statistically significant ($P > 0.05$) (Fig. 2).

Effects on mineralization

Calcified nodules were observed in the MTA and EMD groups compared with the untreated control group (Fig. 3).

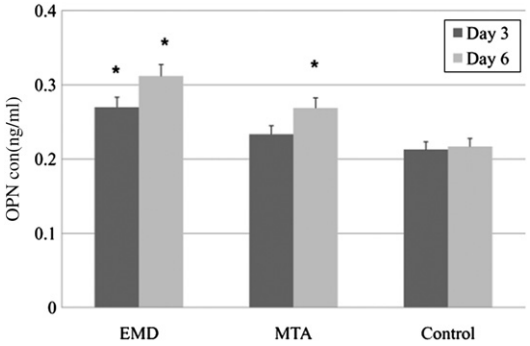

Figure 1 The effects of MTA and EMD on OPN level in HCDCs on Days 3 and 6. The bar represents the mean + standard deviation. (*$P < 0.05$, compared with the control). Con = concentrations; EMD = enamel matrix derivative; MTA = mineral trioxide aggregate; OPN = osteopontin.

Discussion

Complete regeneration of the injured periodontium including the cementum, periodontal ligament, and bone is essential for the repair of perforated areas.[14] In numerous *in vitro* studies, MTA was proven to create a biocompatible environment and stimulate mineralization of cementoblasts. MTA, however, like calcium hydroxide causes inflammatory and necrotic changes in adjacent tissues, although to a lesser degree.[15] Because it is commercially available and more economical than other bioactive agents, EMD has recently been applied for endodontic therapy by numerous clinicians. Although there are previous articles reporting the effects of MTA and EMD on murine cementoblast cell lines, the present study is the first to compare the effects of MTA and EMD on the mineralization capacity of HCDCs in the same investigation. HCDCs, when attached to synthetic hydroxyapatite/tricalcium phosphate ceramic and transplanted into immunodeficient mice, form a cementum-like tissue. HCDCs also maintain the potential to form cementum-like tissue after

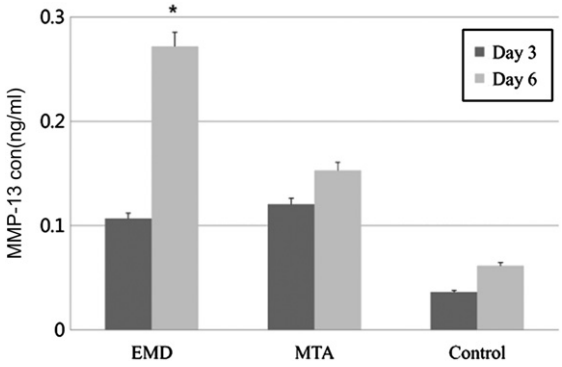

Figure 2 The effects of MTA and EMD on MMP-13 level in HCDCs on Days 3 and 6. The bar represents the mean + standard deviation. (*$P < 0.05$, compared with the control). Con = concentrations; EMD = enamel matrix derivative; HCDC = human cementum-derived cell; MMP = matrix metalloproteinase; MTA = mineral trioxide aggregate.

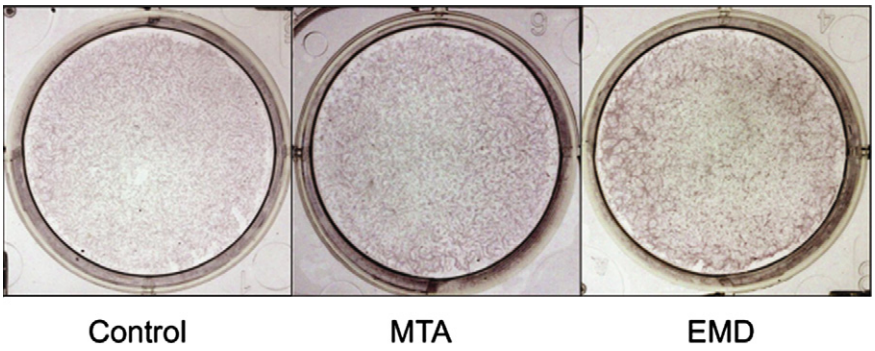

Figure 3 The effects of MTA and EMD on formation of calcified nodules. The HCDCs were cultured with MTA and EMD for 6 days and stained with the Alizarin red. EMD = enamel matrix derivative; HCDC = human cementum-derived cell; MTA = mineral trioxide aggregate.

ex vivo expansion, so HCDCs can be used in studies related to human cementogenesis.[16]

The results for OPN in this study were in accordance with data from previous studies, which found that OPN is upregulated by MTA and EMD in cells with mineralizing potential.[17–19] The increase in OPN expression by MTA and EMD was demonstrated to be time dependent in MG63 cells and murine cementoblasts, respectively.[20,21] Although the experimental period of this study was not long enough to confirm those articles, levels of OPN on Day 6 in both cells with MTA and EMD were higher than those on Day 3. Unlike OPN, few studies evaluated the expression of MMP-13 by MTA and EMD. Although MMP-13's mechanism is not well understood, it was shown to be closely associated with chondrocyte and osteoblast differentiation and also with the formation of a mineralized ECM.[22] Kiili suggested MMP-13's role in periodontal tissue repair and healing by the considerable levels of MMP-13 verified after nonsurgical periodontal treatment.[23] In this study, MMP-13 expression was increased both by EMD and MTA, but the increase by EMD was more distinct on Day 6. EMD was reported to significantly enhance cell proliferation and it was speculated to have affected the level of MMP-13 by increasing cell numbers.[6] Furthermore, Suri et al. reported that MMP-13 showed a striking association with the onset of mineralization in human dental pulp cells and suggested its role as a mineralization marker.[24] The difference in the levels of increase by EMD and MTA might indicate differences in the onset of mineralization induced by EMD or MTA; however, further studies with longer observation periods are needed to prove this. Nevertheless, the present investigation clearly showed that both MTA and especially EMD significantly increased the expressions of OPN and MMP-13 by HCDCs. These results suggest that MMP-13 can be used as a mineralization marker as is OPN, although there should be more investigations analyzing its expression at the RNA level. Mineralized nodules were obviously identified in cells treated with MTA and EMD. It seemed, however, that the experimental period was not long enough to promote sufficient mineralization in untreated HCDCs to assess this aspect.

The increased expressions of OPN and MMP-13 and formation of mineralized nodules all confirm that both MTA and EMD stimulate cementoblastic activity. However, increases in OPN and MMP-13 levels by EMD were higher than those by MTA on Day 6, even though the difference was not significant, which might have been due to the short period of application to cells. Because EMD is more economical than other bioactive agents and seems to show higher accelerating effects on mineralization of HCDCs than MTA as found in the present study, it might be considered a better material for endodontic repair. EMD, however, is supplied in a gel-form, which is a definite limitation on its use; so, there should be further studies to prove the potential of EMD as a repair material at various levels and to overcome limitations to its use.

In conclusion, the findings in the present study confirmed the capacity of both MTA and EMD to accelerate cementoblastic activity and compared with MTA, EMD significantly stimulated mineralization of HCDCs.

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